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PLAQUE TRANSFER ASSAY FOR DETECTING NEUTRALIZING ANTIBODIES TO HTLV-III (AIDS)

Subtitle: HIV-1 Inactivation by Antibodies:
Predominance of a Group-Specific Epitope
that Persists Despite Genetic Variation

Final Report

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The technical objective is to develop a method of determining the number of infectious particles in samples containing the human-T lymphotropic virus type three (HTLV-III) using a blot method with labeled deoxyribonucleotide hybridization or labeled anti HTLV-III antibodies. The neutralization tests and will allow testing for neutralizing antibody in military populations.

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FOREWORD

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

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Summary:

We have analyzed the antibody sensitivity of three divergent
isolates of HIV-1 in a new plaquing assay that detects human
retroviruses as discrete, macroscopic plaques. Because each
plaque derives from a single viral infection event, the plaques
are highly sensitive to inactivation by neutralizing antibodies.
Sera of infected asymptomatic patients were tested for
neutralizing activity against one, two or all three viral
isolates, to determine neutralizing titer and specificity. All
infected patients made neutralizing antibodies, and the
predominant antibodies were group-specific, as defined by the
ability to neutralize divergent strains of HIV-1 equally. All 3
viruses shared one or more group-specific neutralizing epitopes,
in spite of the high degree of genetic diversity and distant
geographic origins among them. None of the three isolates showed
significant antigenic drift or even a modest frequency of antibody
resistant variants. The apparent lack of antibody resistance
among otherwise divergent viruses suggests that HIV-1, by evading
neutralizing antibodies during most of its life cycle, may have
lost an important mechanism for selecting antibody resistant
variants from a large pool of random mutants. These findings may
be relevant to vaccine design, if it becomes possible for vaccine
antigens to elicit similar group-specific neutralizing antibodies
prior to infection.

Introduction

Human immunodeficiency virus type 1 (HIV-1) is the causative agent of acquired immunodeficiency syndrome (1, 2). Despite rapid progress in the isolation (3), cloning and sequencing of the entire viral genome (4-6), relatively little is known about what constitutes a protective immune response to the virus (7). For this reason, we have recently developed a sensitive plaque-forming assay for HIV-1 and have found it to be a sensitive and specific measure of viral inactivation by neutralizing antibodies.

Generally, the strength of plaquing assays is the ability to divide the infection of a monolayer culture into individual molecular infection events, followed by signal enhancement due to exponential growth of the virus. This permits detection of small numbers of viruses and facilitates comparisons between different viruses, since each is based on the same multiplicity of infection (1 per infected cell). It also provides a measure of the initial infection event that is virus-based rather than cell-based. Thus, reduction in the number of infectious viruses due to neutralizing antibodies causes a corresponding and marked reduction in the number of plaque forming units (PFU) detected (8, 9).

Previous efforts at developing a plaquing assay for HIV-1 were thwarted by the lack of a suitable monolayer cell that was both adherant to plastic and capable of supporting the growth of HIV-1. Recently, a CD4+ HeLa cell line constructed by Maddon and Axel (10) was shown to support the growth of HIV-1. We now report the use of this adherant cell line to form a suitable monolayer

for growing HIV-1 as plaques. Viral plaques are detected by transferring the infected monolayer to nitrocellulose, where zones of viral RNA (plaques) are labeled by hybridization with a P32 labeled virus-specific DNA probe (11). Thus, we can detect plaques even when no microscopic changes such as cell fusion or cytopathic effects can be reliably observed.

Using this new plaquing method, we have measured neutralizing antibodies to HIV-1 with a high degree of sensitivity and specificity. We find that three HIV-1 isolates share a common neutralizing epitope, in spite of highly divergent sequences. Nearly all infected patients make antibodies that recognize this site (or sites) and neutralize the laboratory isolates of HIV-1 in a group-specific manner (12). Finally, in spite of a high frequency of base substitutions in HIV-1, we find that antibody-resistance is quite rare, perhaps due to lack of the selective pressure needed for antigenic drift to occur. This assay method has also been successful in measuring viral inactivation by other mechanisms, such as binding to soluble CD4 receptor protein (13-15). Thus, it may be useful in the search for vaccines and other biologic agents that can alter the infectivity and replication of HIV-1.

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Materials and Methods

Cells. The adherent CD4+ HeLa human epithelial carcinoma cell line T4Ps5 (10) was generously provided by Drs. P. Maddon and R. Axel (Columbia University, New York). This line was constructed by transforming adherant HeLa cells with the cloned gene for CD4 and was shown to support the growth of HIV-1 (10). Thus, it formed a monolayer which was suitable for detecting HIV-1 plaques. The cells were were maintained in the presence of C.5 mg/ml Geneticin (G418 sulfate, Gibco, Brand Island, NY) in complete medium containing Dulbeco's Modified Eagle's Medium (DMEM) supplemented with 4.5 g/l glucose and with 15% heat inactivated fetal calf serum, 100 U/ml penicillin, 100 ug/ml streptomycin and 2 mM glutamine. Uninduced cells expressed surface CD4 on about 15% of cells (as measured by cytofluorometry), which increased to 30% after induction with epidermal growth factor (EGF) 100 ng/ml for 1 hour. All assays were done with induced cells, except as noted. For use in the plaquing assay, CD4+ HeLa cells were grown to confluence in a 75 cm^2 flask (about 10^7 cells), harvested with a rubber policeman, washed once, and resuspended in 5 ml of medium, and 30 microliter were added per culture.

Sera. Human sera were obtained from asymptomatic donors who were discovered to be seropositive by routine blood bank screening. They were confirmed positive by demonstrating reactivity to multiple bands on Western blot and were a gift of Dr. J. Wai-Kuo Shih of the NIH Department of Transfusion Medicine. Normal sera were obtained from laboratory workers, and three additional sera counted in this group were false positives by ELISA testing but

were repeatedly Western blot negative over a period of 6 months. Sera from patients with clinical AIDS were a gift of Dr. Robert Yarchoan of the NIH, and sera from patients with adult T cell leukemia, seropositive for HTLV-I, were a gift of Dr. Thomas Waldmann, NIH. Sera from patients with autoimmune diseases were a gift from Dr. Laurence Rubin, University of Toronto Medical School.

Virus. H9 cells infected with the IIIB (5) and RFII (6) isolates of HIV-1 as well as the NIHZ isolate of HIV-2 (17) were provided by Drs. Robert Gallo and Howard Streicher (NCI) and by Drs. Hiroaki Mitsuya and Sam Broder (NCI), and A3.01 cells (18) infected with Z84 (19) were provided by Dr. Thomas Folks (NCI). Virus was passaged monthly by freshly infecting H9 cells, as confirmed by the appearance of viral p24 antigen in the culture supernatants (20). Infected H9 cells were irradiated (10,000 rad, Cs137 irradiator) before use.

Virus-specific DNA Probe. The probe was a slightly truncated 9.2 kB fragment of the cloned BH10 isolate of HIV-1 (5) and was provided by Dr. Chad Giri (Center for Biologics, FDA). It was P32 labeled by nick translation of the viral DNA with a kit from New England Nuclear (Boston, Mass.), followed by separation of the products on a Sephadex G50 spin column, giving a specific activity of 2 X 10^7 cpm/ug. As shown in Fig. 1, the probe was able to detect a dot containing the amount of viral RNA in 5,000 infected H9 cells but there was no background signal from 10^6 uninfected H9 cells. In subsequent experiments, with acutely infected H9 cells, dots containing as few as 2,000 infected cells were detectable.

Plaque-forming assay. The assay can be divided into a virus growth step, followed by a plaque detection step (Fig. 2). CD4+ HeLa cells were incubated for 1 hour in the presence of EGF 100 ng/ml (Collaborative Research, Inc., Bedford, Mass.) followed by polybrene 10 mcg/ml. They were then washed and infected by incubation with various dilutions of cell-free HIV-1 or with various numbers of infected H9 cells for 2 hours at 37'. The cells were diluted to 3 ml and plated in 5 cm tissue culture dishes (Costar, No. 3060) in complete medium (without geneticin). The next day, the culture medium was replaced with an agarose overlay containing 4 ml of 0.6% Seaplaque agarose (Marine Colloids Inc., Rockland, Me.) in complete medium. The cells in the culture dishes were diluted about 1:80 relative to confluence, and they returned to confluence in about 7 days.

If zones of viral infection are formed on the monolayer in the first step, then they are detected in the second step by DNA-RNA hybridization. When the cells of the infected CD4+ HeLa monolayer reached confluence, the entire monolayer was transfered from plastic to nitrocellulose. The agarose overlay was removed from the culture dish and the monolayer was washed with phosphate buffered saline. Pre-cut circles of nitrocellulose paper, saturated with 0.05 M Tris-HCl pH 7 and 0.15 M NaCl were placed on the cell monolayer surface and pressed down firmly enough to make contact, but carefully to avoid smudging. The discs were lifted off the plastic and placed cell side up on a filter paper saturated with 6% formaldehyde in 6 X SSC (0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) and 50% formamide, and the cells were lysed at 65' for 10 minutes. The discs were transfered to filter papers saturated with 6X SSC and 0.1% SDS for 10 minutes followed by 6X SSC for 10 minutes, both at room temperature. The discs

were air dried, interleaved with Patapar paper (Schleicher and Schuell, Keene, NH) and baked in a vacuum oven for 1 hour at 80°C.

After baking, the discs were washed in 6X SSC at 65° for 30 minutes and then placed in a sealed plastic bag with prehybridization mix containing 6X SSC, 10% dextran sulfate, 5% Denhardt's Solution, 0.1% SDS, 0.1% Poly adenosine (>100,000 MW), and 300 ug/ml herring sperm DNA (boiled before use). After prehybridizing 4 hours at 65°, the discs were hybridized with a P32 labeled viral probe in fresh prehybridization mix for 18 hours at 65°.

After hybridizing with the probe, the filters were washed briefly at room temperature with 2X SSC with 1% SDS and then twice at 55° for 30 minutes in the same buffer. After the second wash, there was usually no residual radioactivity in the wash buffer, and the filters were washed a final time in 0.2 X SSC with 0.1% SDS at 50° C for 30 minutes. The discs were then dried and placed in a photographic cassette with X-ray film.

Detection of neutralizing antibodies. A known titer of virus or virally infected H9 cells containing between 100 and 3,000 plaque forming units of HIV-1 was incubated for 2 hours at 37° in the presence of various dilutions of serum from seropositive patients or immunized rabbits in a final volume of 50 ul. This material was diluted to 0.3 ml, and aliquots of 100 ul or 30 ul were used to infect 30 ul of CD4+ HeLa cells by incubating together for 2 hours at 37°, followed by culturing the infected cells in the standard plaquing assay. The neutralizing titer was defined as the serum dilution giving a 50% reduction in plaque number. In addition, each serum was tested at antibody excess to determine the frequency of spontaneously occurring antibody-resistant variants in each viral stock.

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Plaque Formation. Previous attempts at detecting HIV-1 plaques were stymied by the lack of an adherant monolayer cell able to support the growth of the virus and by difficulty visualizing the cytopathic effects of the virus. We have now solved the monolayer problem by use of the genetically engineered CD4+ HeLa cell provided by Maddon and Axel (10). In addition, we have developed an HIV-specific detection system that is sufficiently sensitive to detect the amount of virus in a single plaque. Our approach was to use a modification of the colony hybridization method described by Villareal and Berg (11), using a P32 labeled HIV-1 specific probe. In this way, we could detect plaques form recombinant vaccinia virus that contained the HIV-1 env gene (a gift of Dr. Bernard Moss (21)), but not from wild type vaccinia virus, and the number of plaques detected by hybridization agreed with the number of lytic plaques detected by staining the monolayer with neutral red dye (not shown). Thus, our P32 labeled HIV-specific probe could detect the number of viral copies in a single recombinant vaccinia plaque.

We then used the hybridization method to detect infection of the CD4+ HeLa monolayer by HIV-1. After 7 to 10 days in cullture, we detected discrete, macroscopic viral plaques by hybridization, as shown in Fig. 3. The plaques were 0.5 to 1.5 mm in size, and the plaque number was readily determined when the exposed film was displayed over an x-ray view box. The number of plaques was proportional to the input of virus, and no plaques were formed in the absence of virus. Microscopic observation of the monolayers revealed occasional bizarre forms, including some giant cells, but, at the low multiplicity of infection used in

expression of viral genes following the infection event. By
counting the number of infection events (PFU), we could
distinguish between these two mechanisms. As shown in Fig. 5,
the slope of antigen production vs. PFU remained constant in
spite of EGF activation, indicating that the ratio of antigen
produced per plaque remained constant. Thus, EGF treatment of
the host cell gave more infection events, each of which produced
the same amount of viral antigen per plaque. Increased plaquing
efficiency on activated CD4+ HeLa cells may be due to increased
expression of the CD4 receptor by these cells. Alternatively,
increased transcription of early viral genes in activated host
cells may lead to a greater frequency of productive infections
which result in more plaques.

Plaques from divergent isolates of HIV-1. HIV-1 exhibits a high
degree of sequence variation among different viral isolates, and
we wanted to study plaques from a variety of divergent isolates.
As shown in Fig. 6, plaques were detected from 4 different HIV
isolates, including HIV-1 isolates IIIB, RFII, and Z84, as well
as the HIV-2 isolate NIHZ. In the figure, the vertical distance
between isolates corresponds to the number of substitutions
between them (adapted from ref 24) in the third base of each
codon. In spite of numerous sequence differences, each isolate
infected the CD4+ monolayer and produced discrete plaques
detectable by the probe.

Detection of Neutralizing Antibodies. In other experiments we
have found that the number of plaques is linearly proportional to
the input of cell-free virus. This implies that each plaque
results from infection by a single virus, since the number of
double infections would increase with the second power of the

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<u>Clinical</u> <u>Results</u>. Using this method, we have measured the neutralizing activity of 14 sera from asymptomatic seropositive patients on the IIIB isolate (Fig. 9) and 11 sera from patients with clinical AIDS. As controls, we also tested 11 seronegative normal sera, 5 sera from HTLV I infected patients and 12 sera from autoimmune patients. Each serum was tested at the 1:20 and 1:200 dilutions, and the per cent neutralization at 1:200 are shown in Fig. 9. Sera from 11 normal controls gave < 12% neutralization at either 1:20 or 1:200. In contrast, half of the seropositive patients neutralized >50% at a dilution of 1:200. Thus, the median neutralizing titer was 1:200, and some patients had titers as high as 1:2000 and 1:6000 in subsequent assays. These results exceed by over 1 log the highest titers detectable by earlier assay methods (25-27). Of eleven patients with clinical AIDS, 3 out of 4 with Kaposi's sarcoma had similar levels of neutralizing antibodies to those found in asymptomatic patients, while 7 with opportunistic infections had generally lower levels of neutralizing antibodies.

Sera from 12 autoimmune patients and five patients with antibodies to HTLV-I also failed to neutralize HIV-1 at either dilution. Thus, neutralizing antibodies measured in this assay could distinguish between two members of the human T cell retrovirus family. However, when 7 of the asymptomatic seropositive patients were retested on a second HIV-1 isolate (RFII) they all neutralized IIIB and RFII equally, indicating that they recognize a group-specific neutralizing epitope for HIV-1 (12).

Frequency of Antibody-Resistant Variants. The neutralization assay provides useful information both at antibody dilutions causing 50% inactivation, which gives the antibody titer, and at antibody dilutions causing 99% inactivation. Under conditions of antibody excess, the per cent of virus surviving provides a measure of spontaneously occurring antibody-resistant variants in each isolate. As shown in Fig. 7 and 8, both IIIB and Z84 contained fewer than 1 resistant plaque in 200, while RFII was <1 in 100. In addition, the kinetics of inactivation of IIIB were previously reported to be linear over the first two logs of inactivation (12), indicating that the entire IIIB isolate is uniformly sensitive to antibody. Thus, in spite of extensive molecular variation (24), antibody-resistant variants of HIV-1 are actually quite rare.

Effect of soluble CD4. Recent reports have demonstrated that soluble recombinant CD4 can neutralize HIV-1 in vitro (13-15), as shown by the inhibition of viral gene expression. With the plaquing assay we could determine whether the antiviral effect of CD4 occurred during the initial infection or during subsequent steps of the virus life cycle. As shown in Fig. 10, incubation of virus with soluble recombinant CD4 for 2 hours prior to infection of the monolayer cells gave a marked reduction in plaque number at CD4 concentrations of 1 ug/ml or greater. Thus, CD4 inhibits the number of infection events, perhaps by mimicry of the cellular receptor for HIV-1 or by competition with the receptor. These results also demonstrate that the plaquing assay provides a sensitive measure of viral inactivation, regardless of the mechanism of inactivation.

Discussion

We have developed a plaque forming assay for HIV-1 that provides a sensitive and specific measure of viral inactivation by neutralizing antibodies. As demonstrated originally with antibody mediated inactivation of haptenated bacteriophage (8), the exquisite sensitivity of PFU to neutralizing antibodies is due to the fact that each plaque results from infection by a single virus, so inactivation of even a few viruses can be detected. Because this assay measures a subset of antibodies with biological activity against the virus, it may be selective for just those antibodies that are relevant to the host's immune response to HIV-1 infection or vaccination.

The specificity of the neutralizing assay comes from the fact that only a minority of the antibodies that bind to a virus can inactivate it. Only the subset of antibodies that bind to neutralizing epitopes cause viral inactivation, and these are quite specific for each virus (28). For example, HTLV-I, which partially cross reacts with anti-HIV-1 antibodies in a Western blot (29), fails to elicit neutralizing antibodies to HIV-1. However, it is also common for different isolates of the same virus family to share a group-specific neutralizing epitope, as shown previously for HIV-1 (12, 30 - 32).

By comparing the sensitivity of two divergent virus isolates to the same antibody, we have recently demonstrated group-specific antibodies, which neutralize both isolates, in the sera of each of 7 asymptomatic seropositive patients (12). Both the IIIB and RFII isolates were neutralized equally, despite sequence differences of 21% in the amino acid sequence of the envelope proteins (6). Similarly, in the present paper, we have extended

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these results to include a third isolate Z84, which is even more divergent in sequence and more distant in geographic origin (19). Since each patient was probably infected with yet another non-laboratory strain of HIV-1, these results suggests that a major group-specific epitope (or epitopes) is widely shared among HIV-1 isolates, including laboratory and feral strains from two continents. Our results also indicate that the group-specific epitope is immunogenic in nearly all infected patients.

The plaque assay provides a sensitive digital readout of the number of infectious viruses in a sample. Only with such an assay could we analyze the antibody sensitivity of hundreds of viral plaques at a time, in order to detect rare variants. In the presence of excess neutralizing antibodies, the nearly complete inactivation of all PFU indicated that fewer than 0.5% of the viruses in each isolate were spontaneously antibody-resistant, and the actual frequency may be much lower.

Superficially, these results appear to contradict the known high frequency of molecular variation among different HIV-1 isolates. However, genetic variation is also quite common among other RNA viruses, including some viruses which display a high tendency for antigenic drift to escape antibodies, such as influenza (33), and other viruses which appear never to drift, such as poliovirus (34). In both cases, mutants are formed frequently due to improper base substitutions, limited only by the nonviability of mutations affecting essential viral functions. But these mutants only serve as the substrate for immunologic selection. Without selection among the randomly formed variants, there is no antigenic evolution toward antibody-resistance. Selection is mainly accomplished when virus tries to

grow in the presence of antibody, but it may not occur when most infections are first time infections or when the virus evades antibodies in other ways, such as remaining in a dormant proviral state during most of its life cycle. Thus, given the rarity of reinfections (35) and the prolonged latency of integrated virus, it is not surprising that numerous molecular variants of HIV-1 can coexist without evolving toward antibody resistance. Our data contradict some of the evidence on antigenic drift based on bulk cultures (36), but agree with stronger observations based on plaques (37) and pseudoplaques (30).

Other assay methods for detecting HIV-1 neutralizing antibodies are available. These include the VSV pseudotype assay (25), immunofluorescence counting of infected cells (26), syncytium formation (27), trans activation of an endogenous LTR-CAT marker (38) and infection of a CD4+ monolayer detected by monoclonal antibodies (37). Methods requiring microscopic observation and interpretation tend to be tedious and subjective, and are often carried out at high multiplicity of infection so that a visible change will occur, while the pseudotype method can only measure antiviral effects specific for the envelope protein and only at the start of infection. In general, it is preferable to measure the number of infectious viruses rather than the number of infected cells. This makes it possible to divide the infection into individual infection events at a low multiplicity of infection, as we have done, and to attain maximal antibody sensitivity.

Our results with patient sera suggest that neutralizing antibodies may have biological relevance to the spread and prevention of HIV-1 infection. Nearly all infected patients produced neutralizing antibodies. These antibodies were

generally capable of recognizing a group-specific epitope that is shared among divergent HIV-1 isolates such as IIIB, RFII, and Z84. The group-specific epitope was also widely shared among the pathogenic strains that infected our patients, since the various strains that infected 7 patients all elicited group-specific antibodies (12). The group-specific epitope was also broadly immunogenic, since nearly every infected patient responded to it. The general correlation between a patient's titer of neutralizing antibodies and his stage of disease, whether asymptomatic seropositive, Kaposi's sarcoma, or opportunistic infections could be either cause or effect. The predictive value of high titered neutralizing antibodies is currently unknown, and most infected patients progress to clinical immunodeficiency in spite of having neutralizing antibodies. However, the prolonged duration of the asymptomatic infected state (39) may be due to neutralizing antibodies, and the rarity of reinfection (33) may also be due to group-specific immunity. Thus, the clinical evidence is consistent with the hypothesis that neutralizing antibodies might protect against HIV-1 infection if elicited by a vaccine antigen prior to exposure to virus. According to this hypothesis, a reasonable intermediate goal for noninfectious vaccine antigens would be to induce neutralizing antibodies equivalent to those elicited by infection with live virus.

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References

1. Barre-Sinoussi, F., Chermann, J.C., Rey, F., Nugeyre, M.T., Charmaret, S., Gruest, J., Dauguet, C., Axler-Blin, C., Vezinet-Brun, F., Rouzioux, C., Rozenbaum, W., and Montagner, L. (1983) Isolation of a T lymphotrophic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science 220, 868-871.

2. Popovic, M., Sarngadharan, M.G., Read, E., and Gallo, R.C. (1984) Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. Science 224, 497-500.

3. Gallo, R.C., Salahuddin, S.Z., Popovic, M., Shearer, G.M., Kaplan, M., Haynes, B.F., Palker, T.J., Redfield, R., Oleske, J., Safai, B., White, G., Foster, P., and Markham, P.D. (1984) Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. Science 224:500-503.

4. Ratner, L., Haseltine, W., Patarca, R., Livak, K.J., Starcich, B., Josephs, S.F., Doran, E.R., Rafalski, J.A., Whitehorn, E.A., Baumeister, K., Ivanoff, L., Petteway, S.R., Jr., Pearson, M.L., Lautenberger, J.A., Papas, T.S., Ghrayeb, J., Chang, N.T., Gallo, R.C., and Wong-Staal, F. (1985) Complete nucleotide sequence of the AIDS virus, HTLV-III. Nature 313, 277-284.

5. Sanchez-Pescador, R., Power, M.D., Barr, P.J., Steimer, K.S., Stempien, M.M., Brown-Shimer, S.L., Gee, W.W., Renard, A., Randolph, A., Levy, J.A., Dina, D., Luciw, P.A. (1985) Nucleotide sequence and expression of an AIDS-associated retrovirus (ARV-2). Science 227, 484-492.

6. Starcich, B.R., Hahn, B.H., Shaw, G.M., McNeely, P.D., Modrow, S., Wolf, H., Parks, E.S., Parks, W.P., Josephs, S.F., Gallo, R.C., and Wong-Staal, F. (1986) Identification and characterization of conserved and variable

regions in the envelope gene of HTLV-III/LAV, the retrovirus of AIDS. Cell
<u>45</u>, 637-648.

7. Skinner, M.A., Langlois, A.J., McDanal, C.B., McDougal, J.S., Bolognesi,
    D.P., and Matthews, T.J. (1988) Neutralizing antibodies to an immunodominant
    envelope sequence do not prevent gp120 binding to CD4. J. Virol. 4195-4200.

8. Haimovich, J., Hurwitz, E., Novik, N., and Sela, M. (1970) Preparation of
    protein-bacteriophage conjugates and their use in detection of anti-protein
    antibodies. Biochim. Biophys. Acta. <u>207</u>:115-124.

9. Dulbecco, R., Vogt, M., and Strickland, A.G.R. (1956) A study of the basic
    aspects of neutralization of two animal viruses, Western equine encephalitis
    virus and poliomyelitis virus. Virology <u>2</u>, 162-205.

10. Maddon, P., Dalgleish, A.G., McDougal, J.S., Clapham, P.N., Weiss, R.A.,
    Axel, R. (1986) The T4 gene encodes the AIDS virus receptor and is express
    in the immune system and the brain. Cell <u>40</u>:93-104.

11. Villarreal, L.P. and Berg, P. (1977) Hybridization in situ of SV40 plaques:
    Detection of recombinant SV40 virus carrying specific sequences of nonviral
    DNA. Science <u>196</u>:183.

12. Berkower, I., Murphy, D., Wai-Kuo Shih, J., and Giri, C. A major group-
    specific neutralizing epitope of HIV-1 is broadly immunogenic in infected
    patients, submitted.

13. Smith, D.H., Byrn, R.A., Marsters, S.A., Gregory, T., Groopman, J.E., and
    Capon, D.J. Blocking of HIV-1 infectivity by a soluble, secreted form of
    the CD4 antigen (1987) Science <u>238</u>:1704-1707.

14. Fisher, R.A., Bertonis, J.M., Meier, W., Johnson, V.A., Costopoulos, D.S., Liu, T., Tizard, R., Walker, B.D., Hirsch, M.S., Schooley, R.T., and Flavell, R.A. HIV infection is blocked in vitro by recombinant soluble CD4 (1988) Nature 331:76-78.

15. Hussey, R.E., Richardson, N.E., Kowalski, M., Brown, N.R., Chang, H.-C., Siliciano, R.F., Dorfman, T., Walker, B., Sodroski, J., and Reinherz, E.L. (1988) A soluble CD4 protein selectively inhibits HIV replication and syncytium formation. Nature 331:78-81.

16. Maddon, P.J., Littman, D.R., Godfrey, M., Maddon, D.E., Chess, L., and Axel, R. (1985) The isolation and nucleotide sequence of a cDNA encoding the T cell surface protein T4: A new member of the immunoglobulin gene family Cell 42:93-104.

17. Albert, J., Bredberg, U., Chiodi, F., Boettiger, B., Fenyoe, E.M., Norrby, E., and Biberfeld, G. (1987) A new human retrovirus isolate of West African origin (SBL-6669) and its relationship to HTLV-IV, LAV-II and HTLV-IIIB. AIDS Research and Human Retroviruses 3:3-10.

18. Folks, T., Benn, S., Rabson, A., Theodore, T., Hoggan, M.D., Martin, M., Lightfoote, M., and Sell, K. (1985) Characterization of a continous T cell line susceptible to the cytopathic effects of the acquired immunodeficiency syndrome (AIDS)-associated retrovirus. Proc. Natl. Acad. Sci. 82:4539.

19. Benn, S., Rutledge, J., Folks, T., Gold, J., Baker, L., McCormick, J., Feorino, P., Piot, P., Quinn, T., and Martin, M.A. (1985) Genomic heterogeneity of AIDS retroviral isolates from North America and Zaire. Science 230:949.

20. McDougal, J.S., Cort, S.P., Kennedy, M.S., Cabridilla, C.D., Feorino, P.M., Francis, D.P., Hicks, D., Kalyanaraman, V.S., and Martin, L.S. (1985) Immunoassay for the detection and quantitation of infectious human

retrovirus, lymphadenopathy-associated virus (LAV). J. Immunol. Meth. 76:171-183.

21. Chakrabarti, S., Robert-Guroff, M., Wong-Staal, F., Gallo, R.C., and Moss, B. (1986) Expression of the HTLV-III ennvelope gene by a recombinant vaccinia virus. Nature 320:535-537.

22. Nabel, G. and Baltimore, D. (1987) An inducible transcription factor activates expression of human immunodeficiency virus in T cells. Nature 326:711-713.

23. Kaufman, J.S., Valandra, G., Roderiquez, G., Bushar, G., Giri, C., and Norcross, M. (1987) Phorbol ester enhances human immunodeficiency virus promoted gene expression and acts on a repeated 10 base pair functional enhancer element. ·Molec. Cell. Biol. 7:3759-3766.

24. Smith, T.F., Srinivasan, A., Schochetman, G., Marcus, M., and Myers, G. (1988) The phylogenetic history of immunodeficiency viruses. Nature 333:573-575.

25. Weiss, R.A., Clapham, P.R., Cheingsong-Popov, R., Dalgleish, A.G., Carne, A.C., Weller, I.V.D., and Tedder, R.S. (1985) Neutralization of HTLV-III, by sera of AIDS and AIDS at risk patients. Nature 316:69-71.

26. Robert-Guroff, M., Brown, M., and Gallo, R.C. (1985) HTLV-III-neutralizing antibodies in patients with AIDS and AIDS-related complex. Nature 316:72.

27. Nara, P.L., Hatch, W.C., Dunlop, N.M., Robey, W.G., Arthur, L.O., Gonda, M.A., and Fishinger, P.J. (1987) Simple, rapid, quantitative, syncytium-forming microassay for the detection of human immunodeficiency virus neutralizing antibody. AIDS Research and Human Retroviruses 3:283-302.

28. Mandel, B.   Interaction of viruses with neutralizing antibodies, in Comprehensive Virology 15:Viral-Host Interactions (ed. Fraenkel-Conrat, H. and Wagner, R.R.) Plenum, New York, 1979, Ch. 2.

29. Essex, M., McLane, M.F., Lee, T.H., Falk, L., Howe, C.W.S., Mullins, J.I., Cabradilla, C., Francis, D.P. (1985) Antibodies to cell membrane antigens associated with human T-cell leukemia virus in patients with AIDS.   Science 220:859-862.

30. Weiss, R.A., Clapham, P.R., Weber, J.N., Dalgleish, A.G., Lasky, L.A., and Berman, P.W. (1986) Variable and conserved neutralization antigens of human immunodeficiency virus.   Nature 324:572-575.

31. Ho, D.D., Sarngadharan, M.G., Hirsch, M.S., Schooley, R.T., Rota, T.R., Kennedy, R.C., Chanh, T.C., and Sato, V.L. (1987) Human immunodeficiency virus neutralizing antibodies recognize several conserved domains on the envelope glycoproteins.   J. Virol. 61:2024-2028.

32. Prince, A.M., Pascual, D., Kosolapov, L.B., Kurokawa, D., Baker, L., and Rubinstein, P. (1987) Prevalence, clinical significance, and strain specificity of neutralizing antibody to the human immunodeficiency virus. J. Infect. Dis. 156:268-272.

33. Gerhard, W., Yewdell, J., Webster, R., and Frankel, M.E. (1981) Antigenic structure of influenza virus haemagglutinin defined by hybridoma antibodies. Nature 290:713-716.

34. Smith, D.B. and Inglis, S.C. (1987) The mutation rate and variability of eukaryotic viruses: An analytical review.   J. Gen. Virol. 68:2729-2740.

35. Saag, M.S., Hahn, B.H., Gibbons, J., Li, Y., Parks, E.S., Parks, W.P., and Shaw, G.M. (1988) Extensive variation of human immunodeficiency virus type-1 in vivo.   Nature 334:440-444.

36. Looney, D.J., Fisher, A.G. , Putney, S.D., Rusche, J.R., Redfield, R.R., Burke, D.S., Gallo, R.C., Wong-Staal, F. (1988) Type-restricted neutralization of molecular clones of human immunodeficiency virus. Science 241:357-359.

37. Chesebro, B. and Wehdy, K. (1988) Development of a sensitive quantitative focal assay for human immunodeficiency virus infectivity. J. Virol. 62:3779-3788.

38. Felber, B.K. and Pavlakis, G.N. (1988) A quantitative bioassay for HIV-1 based on trans-activation. Science 239:184-187.

39. Medley, G.F., Anderson, R.M., Cox, D.R. (1987) Incubation period of AIDS in patients infected via blood transfusion. Nature 328:719-721.

Figures

Figure 1. Detection of viral nucleic acid in infected H9 cells. Different numbers of infected or uninfected cells were blotted onto nitrocellulose paper and viral nucleic acid was detected by hybridization with a P32 labeled probe.

Figure 2. Method for developing HIV-1 plaques. Virus is grown on the CD4+ monolayer for 7 days. Discrete foci of infection are detected by transferring the entire monolayer to nitrocellulose followed by cell lysis, baking to fix viral nucleic acids on the nitrocellulose, and hybridization with a P32 labeled virus-specific probe.

Figure 3. Plaques from cell-free HIV-1. The CD4+ monolayer cells were infected with cell-free supernatants of IIIB-infected H9 cell cultures and assayed for HIV-1 plaques.

Figure 4. Antigen production and PFU were measured for various input amounts of IIIB infected H9 cells. Plaque formation depends on viral infection and replication, which occurs only on a CD4+ monolayer. $(K=10^3)$

Figure 5. EGF activation of CD4+ HeLa cells gave increased PFU and increased production of p24 antigen. CD4+ monolayer cells were infected with 25 X 10^4 (circles), 5 X 10^4 (squares), or 10^4 (triangles) infected H9 cells. Prior treatment of the monolayer cells with EGF is indicated by closed symbols, while the untreated monolayer cells are indicated by open symbols. Constant slope indicates that EGF increased the plaque number without changing the production of viral antigen per plaque.

Figure 6. Plaques of divergent HIV isolates. H9 cells infected with each viral isolate were assayed for plaque formation. The vertical distance between isolates corresponds to third base substitutions in the codons of the envelope gene (adapted from ref 24). (Strain Z84 corresponds to Zaire-1 in ref 19 and has been substituted for Zaire-3, which it resembles, in the figure).

Figure 7. Antibody sensitivity of IIIB and RFII isolates. H9 cells infected with either isolate were incubated with various dilutions of a patient's serum. The surviving plaques were measured in the standard assay. A normal control serum had no effect at the 1:40 dilution.

Figure 8. Antibody sensitivity of IIIB and Z84 isolates. H9 cells infected with either isolate were incubated for 2 hours with the same patient's serum as in Fig. 7 or with a normal control serum. The surviving fraction of virus is V/Vo, where V is the number of PFU surviving in the presence of antibody and Vo is the number of PFU in the absence of antibody. Vo for the untreated controls were: 602 for IIIB and 936 for Z84. PFU were determined in sextuplicate for IIIB and once for Z84. 25

Figure 9. Clinical summary. Each patient's serum was assayed for neutralizing activity at 1:20 and 1:200 dilutions. Symbols are as follows: AIDS patients with Kaposi's sarcoma (closed squares) or with opportunistic infections (open squares); autoimmune patients with systemic lupus (closed circles) or with rheumatoid arthritis or Sjogren's syndrome (open circles). The per cent neutralization of IIIB at the 1:200 dilution is shown for each serum and is calculated by the formula $(1-V/Vo) \times 100$, where V is the number of PFU surviving and Vo is the number of PFU with no antibodies added.

Figure 10. Soluble recombinant CD4 neutralizes HIV-1. Increasing doses of rCD4 were incubated with cell-associated virus for 2 hours at 37^0 followed by infecting the monolayer cells in the presence of the inhibitor for an additional 5 hours. Recombinant CD4 inhibited PFU at concentrations of 1 ug/ml or higher.

FIGURE 1
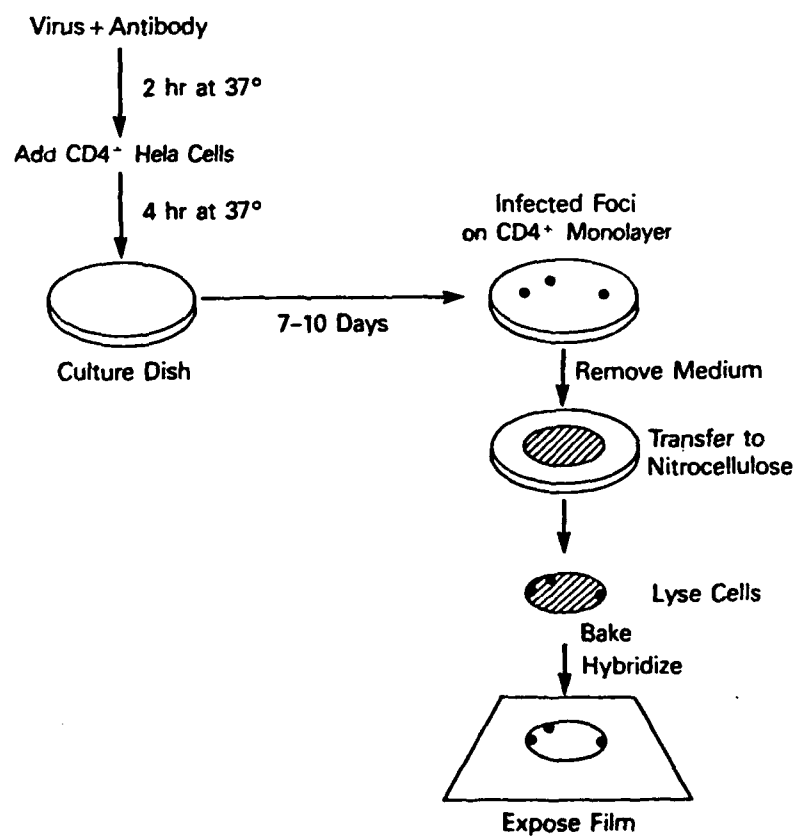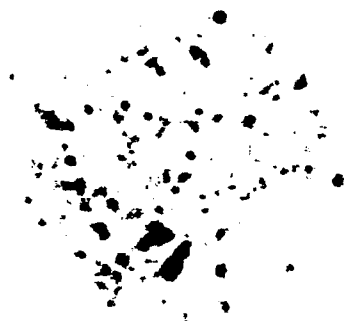
DETECTION OF VIRAL NUCLEIC ACIDS

CELL NUMBER	5K	25K	50K	100K	125K	250K	500K	10^6
Infected	●	●	●	●	●	●	●	●
Uninfected								
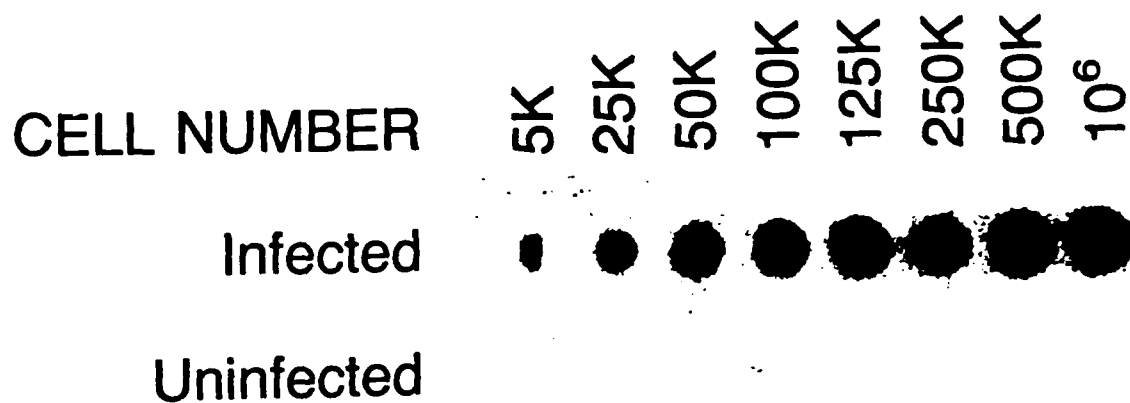
FIGURE 2

Plaque Transfer Method

Virus + Antibody

↓ 2 hr at 37°

Add CD4⁺ Hela Cells

↓ 4 hr at 37°

Culture Dish → 7-10 Days → Infected Foci on CD4⁺ Monolayer

↓ Remove Medium

Transfer to Nitrocellulose

↓

Lyse Cells

Bake
↓ Hybridize

Expose Film

FIGURE 3

Plaque Transfer Assay of Free Virus

Uninfected

100λ HIV

33λ HIV

FIGURE 4

FIGURE 5
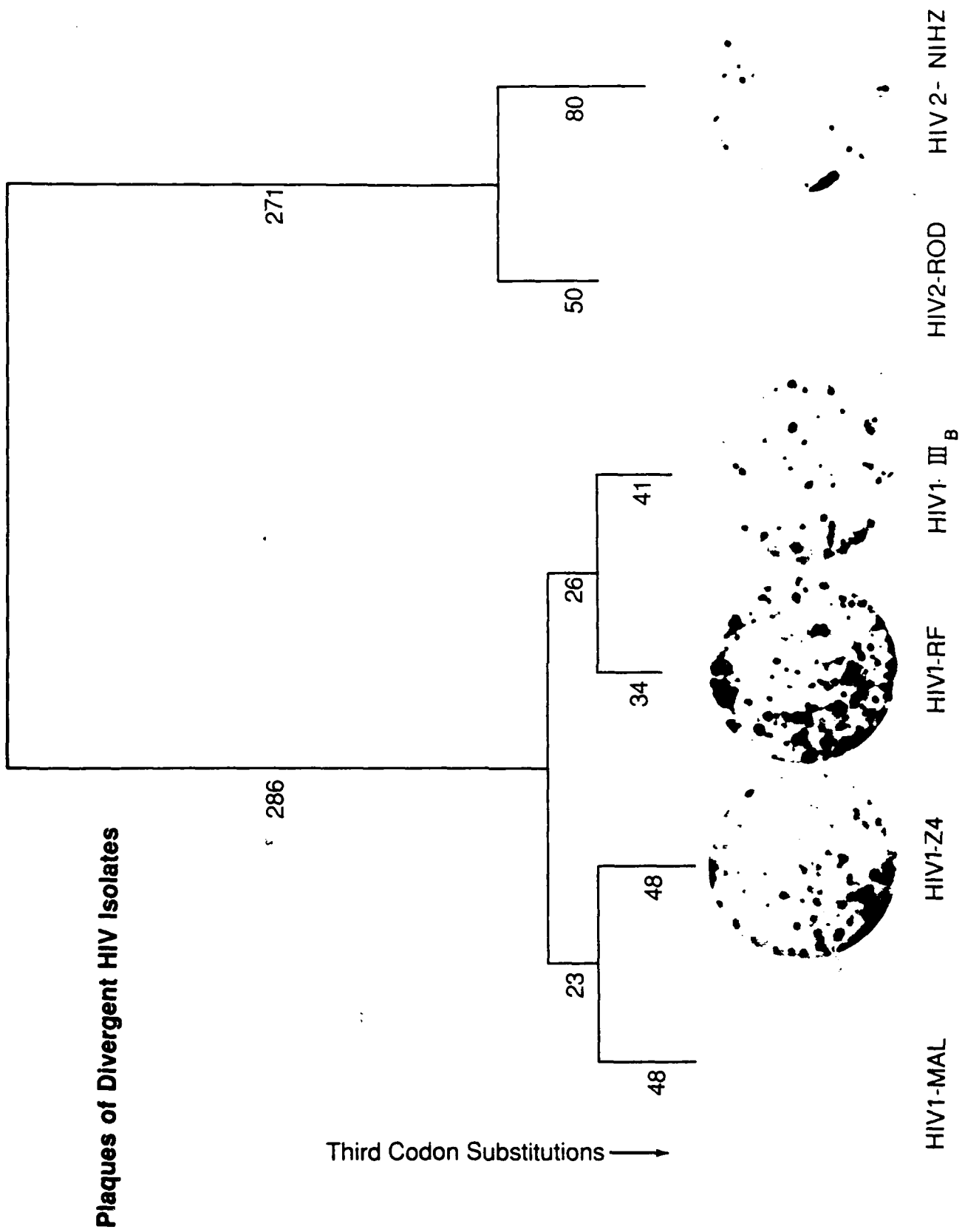

ANTIGEN PRODUCTION INCREASES LINEARLY
WITH THE NUMBER OF PLAQUES

FIGURE 6

Plaques of Divergent HIV Isolates

Third Codon Substitutions ⟶
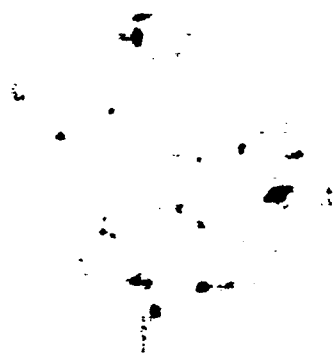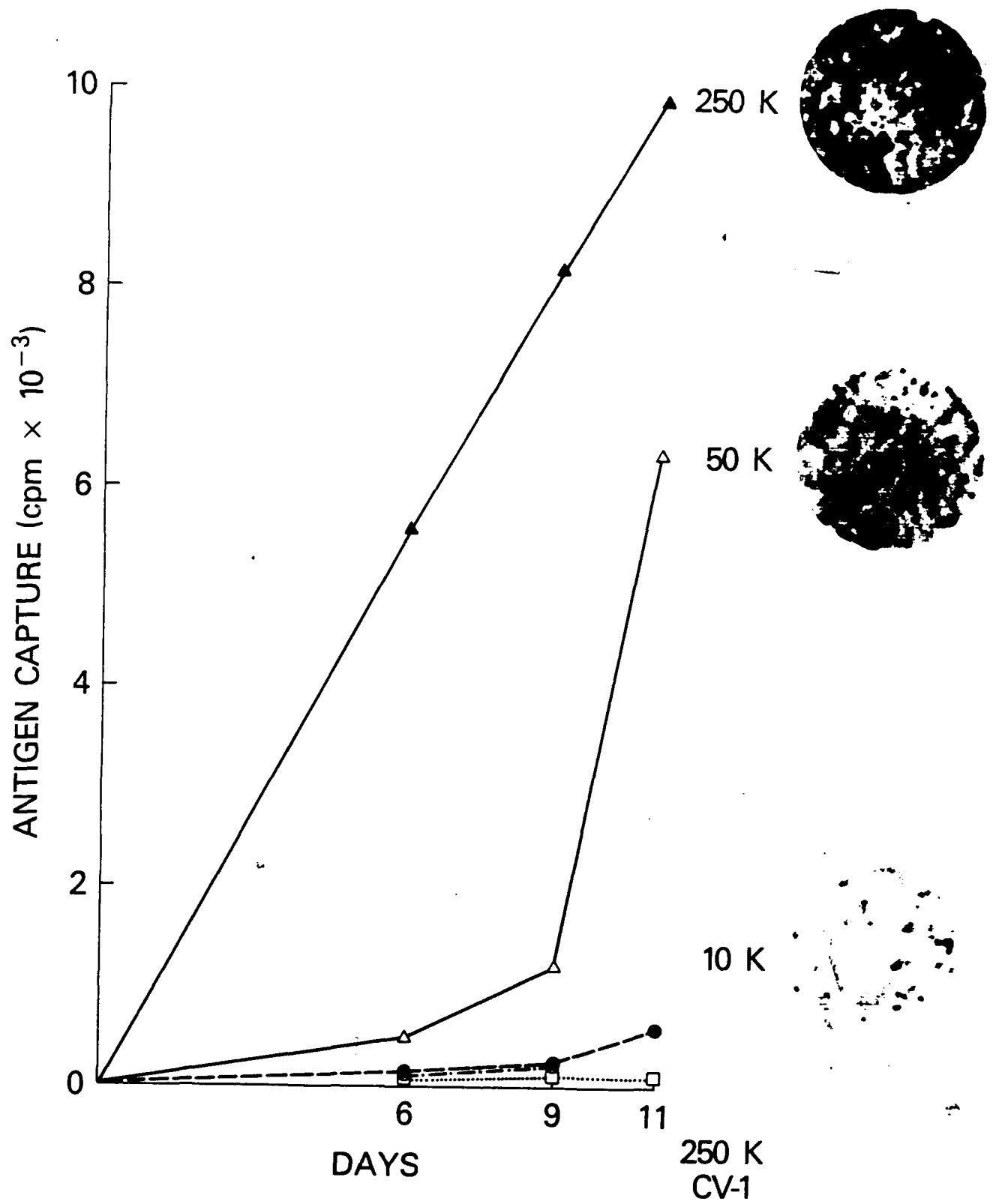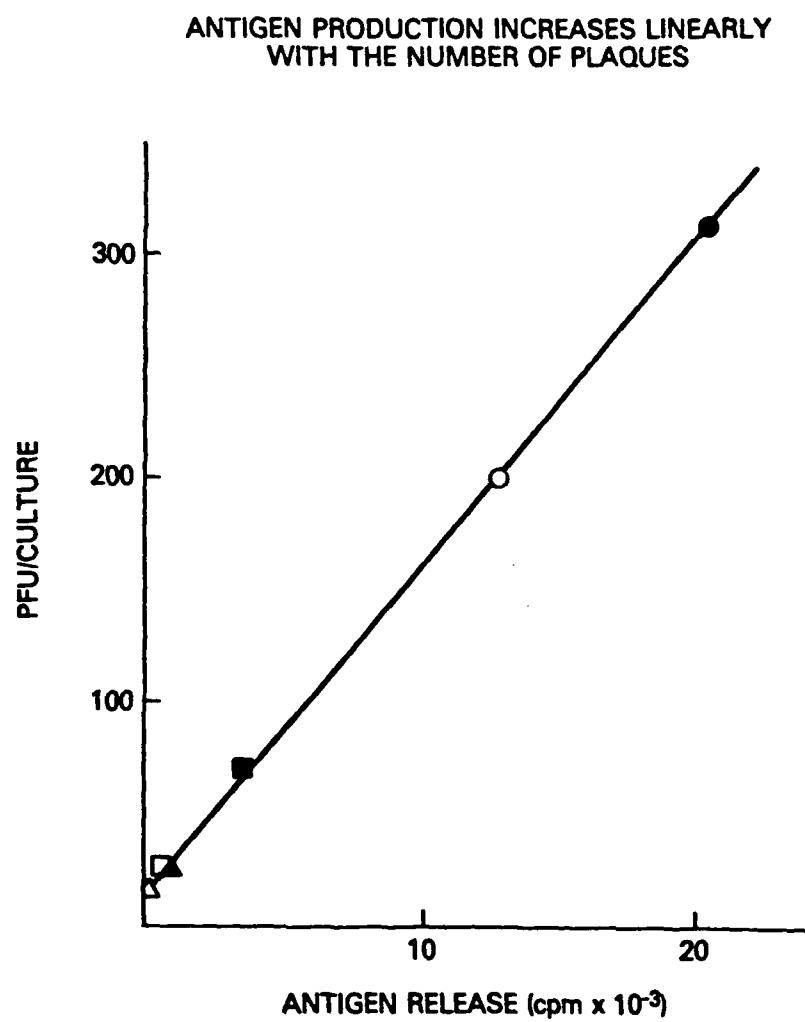
FIGURE 7

HUMAN NEUTRALIZING ANTIBODIES TO HIV-1
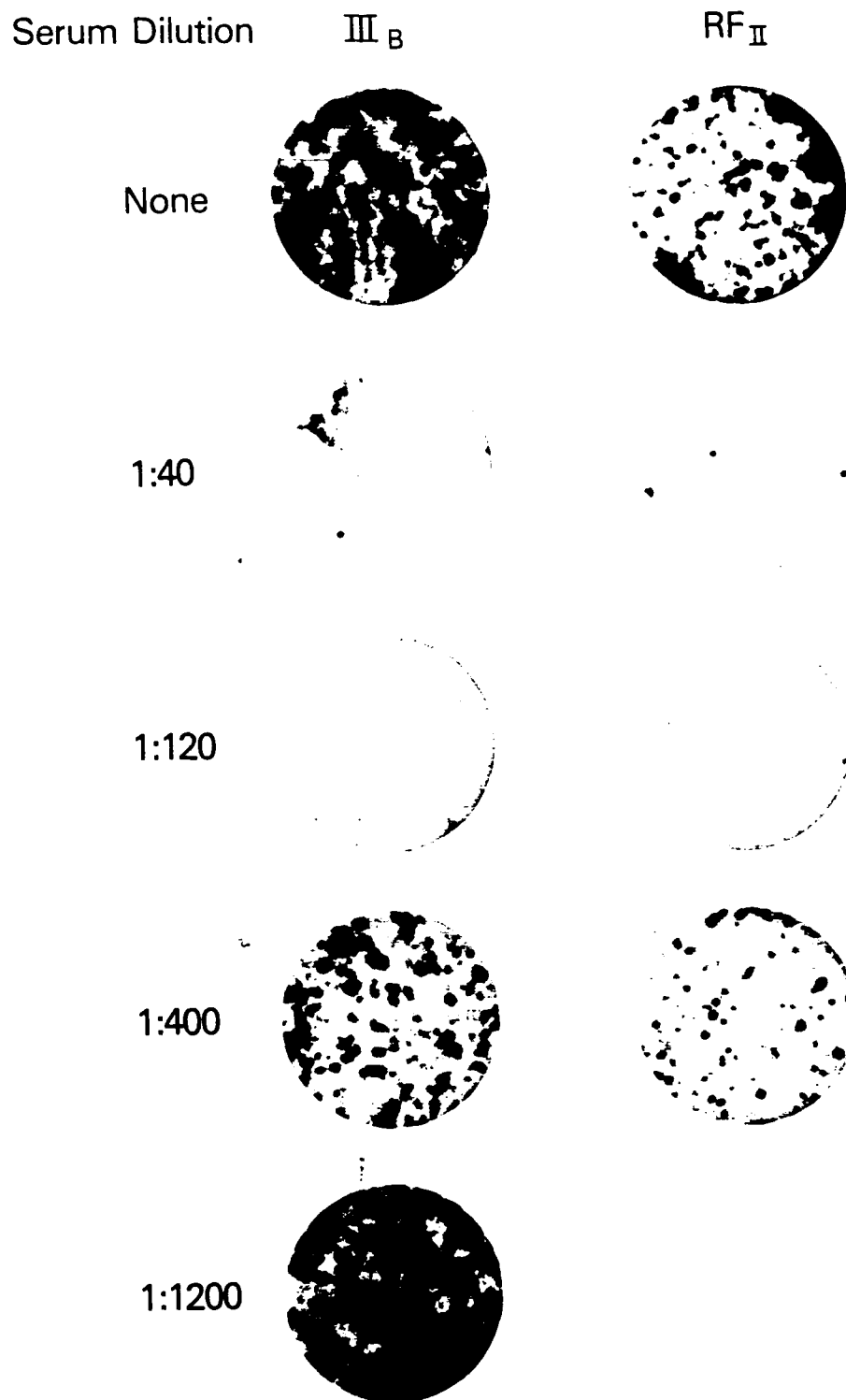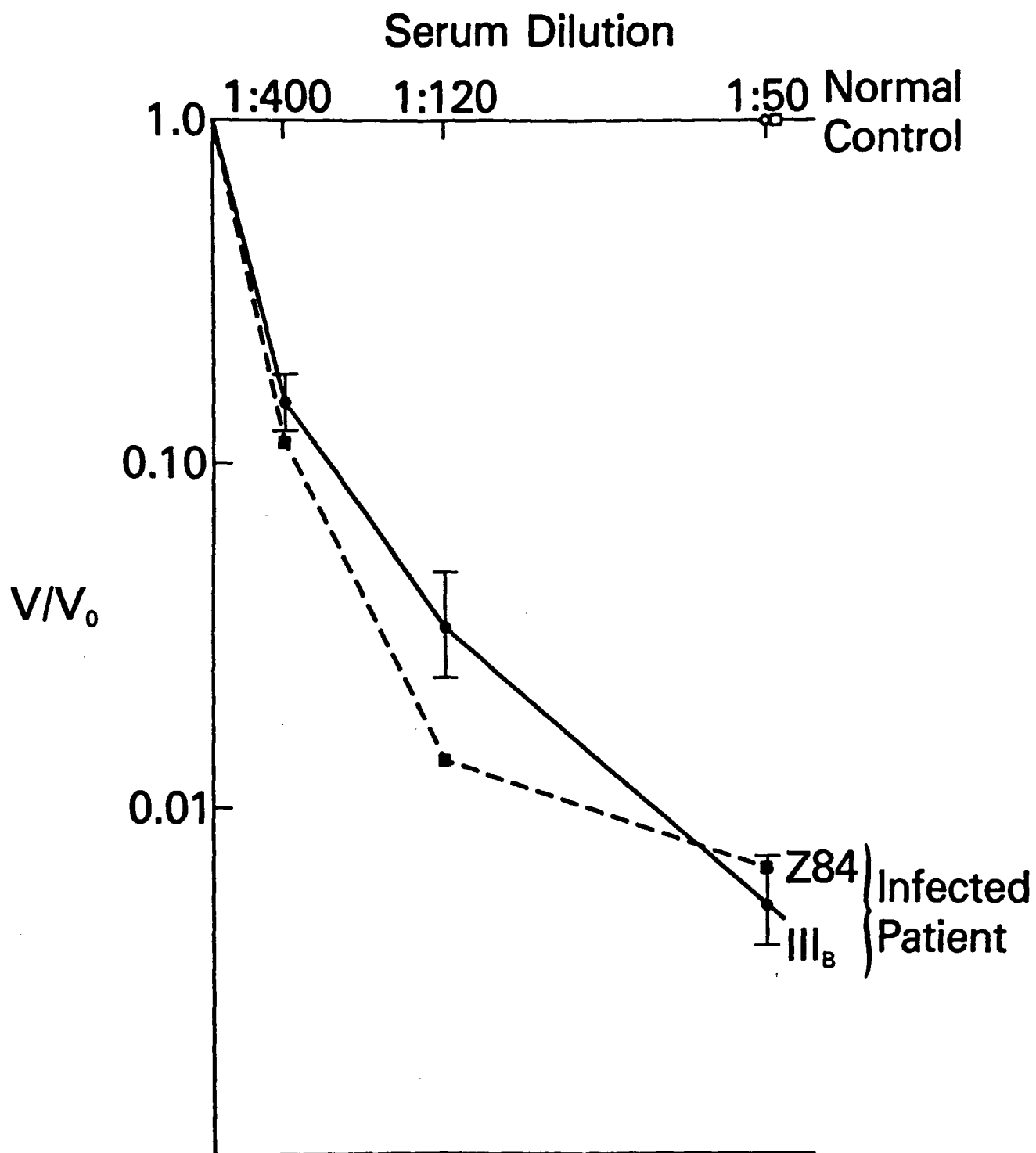
HIV-1 Isolate

FIGURE 8

FIGURE 8
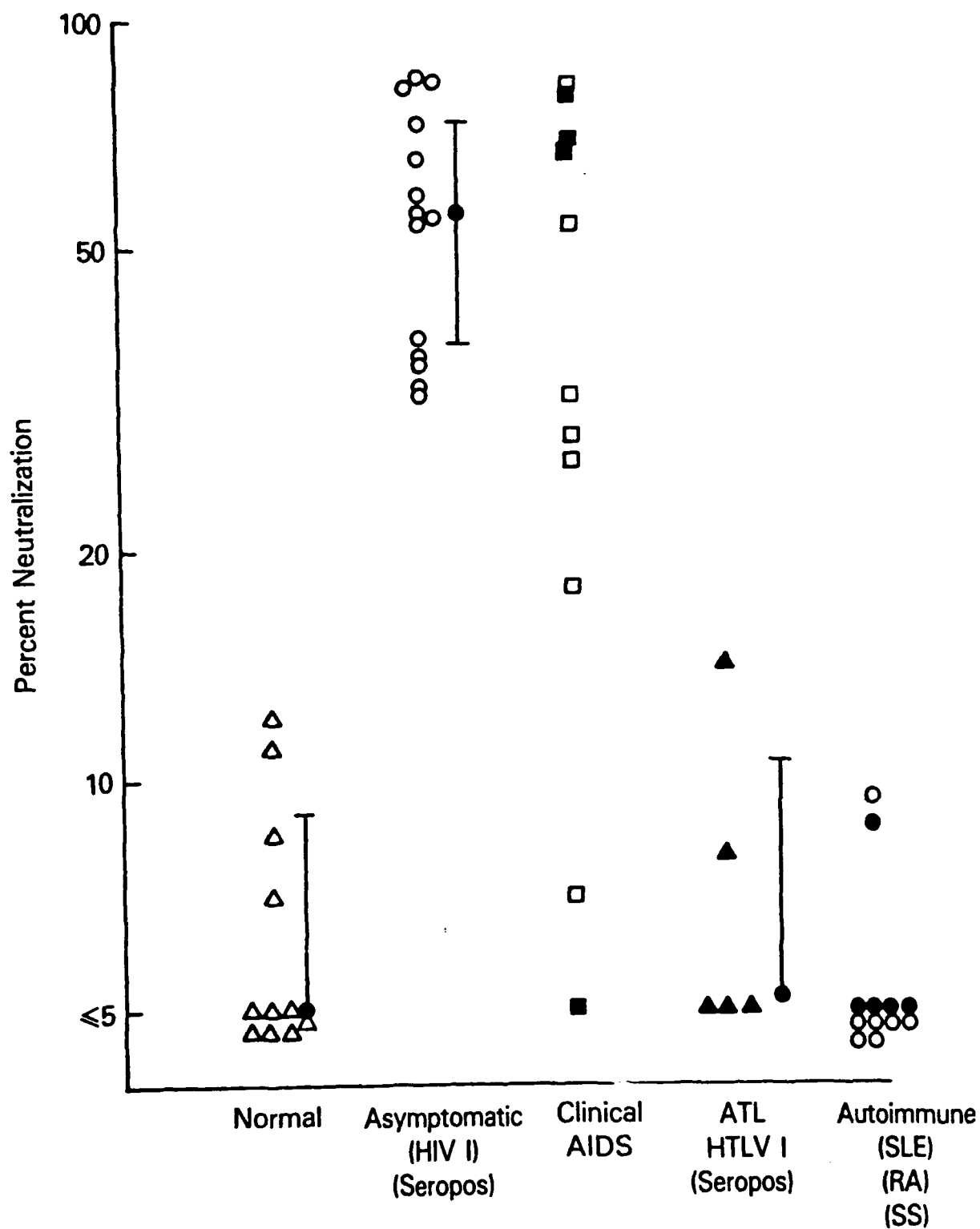
FIGURE 9

HIV-1 Neutralizing Activity in Patients' Sera

FIGURE 10

rCD4 Inhibits HIV-1 Plaques